Interaction of Human Immunodeficiency Virus Type 1 Tat-Derived Peptides with TAR RNA†

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ABSTRACT: Basic peptides from the carboxy terminus of the human immunodeficiency virus type 1 (HIV-1) Tat protein bind to the stem−loop region of TAR RNA, spanning a trinucleotide bulge, with high affinity and moderate specificity. Previous studies have demonstrated that TAR RNA contains a specific arginine binding pocket. A series of 24 amino acid Tat-derived peptides with one or two arginines has been evaluated as possible structural models of the wild-type peptide in its interaction with TAR RNA, using gel electrophoretic methods and circular dichroism (CD) spectroscopy. Dissociation rate measurements indicate that these peptides form complexes with TAR RNA that are significantly less stable kinetically than the wild-type complex. Through a combination of dissociation and association rate measurements, we estimate that wild-type Tat peptide and TAR RNA interact with a K_d of about 16 pM. Together with competition experiments, these results confirm that band shift gel titration methods significantly underestimate absolute peptide−RNA binding affinities in the subnanomolar range. Through competition experiments with bulge mutants of TAR RNA, we demonstrate that peptides that form longer lived complexes with wild-type TAR RNA also show greater discrimination over TAR RNA bulge mutants. Difference CD spectra show that the Tat-derived peptides do not induce the same changes in TAR RNA as the wild-type peptide. The difference CD spectrum of argininamide bound to TAR RNA is most similar to that of the wild-type peptide−TAR RNA complex, implying that the differences in CD spectra upon complex formation are mostly due to changes in TAR RNA conformation.

The human immunodeficiency virus type 1 (HIV-1) Tat protein is a potent transactivator of viral gene expression and is essential for viral replication (Cullen, 1991; Cullen & Greene, 1989; Fisher et al., 1986; Steffy & Wong-Staal, 1991). Tat stimulates transcription after binding to its RNA target, the transactivation response element or TAR RNA, which is found immediately downstream from the site of transcription initiation at the 5′ end of all viral messenger RNA transcripts (Berkhout et al., 1989). Nuclease mapping studies first demonstrated that TAR RNA adopts a hairpin structure, including a hexanucleotide loop, a trinucleotide bulge, several single bulges, and extensive duplex regions (Muesing et al., 1987). Nucleotides +20 through +43 (+1 denoting the transcription start site) of the TAR RNA hairpin are sufficient for transactivation in vivo (Jakobovits et al., 1988). The trinucleotide bulge is necessary for both transactivation and specific binding to Tat (Cordingley et al., 1990; Dingwall et al., 1990; Roy et al., 1990; Weeks et al., 1990). Studies showing that the TAR RNA loop is essential for transactivation (Berkhout & Jeang, 1989; Feng & Holland, 1988) but not for specific Tat binding have led to proposals that the loop may bind a human cellular cofactor involved in the regulation of transcription. Indeed, several proteins that specifically bind the TAR RNA loop have recently been identified (Marciniak et al., 1990; Sheline et al., 1991; Wu et al., 1991).

The Tat protein (BRU isolate; Wain-Hobson et al., 1985) is a small protein consisting of 86 amino acids. Tat residues 1−72 are necessary for optimal interactions in vivo (Frankel et al., 1989; Kuppuswamy et al., 1989). Tat contains a cysteine-rich domain (9 cysteines in 16 residues) and a highly basic region (6 arginines and 2 lysines in 9 residues) (Arya et al., 1985; Sodroski et al., 1985). The cysteine-rich domain contains a binding site for two metal (M^{2+}) ions and may be involved in the metal-linked dimerization of Tat (Frankel et al., 1988). The basic region of Tat is responsible for specific binding to TAR RNA (Cordingley et al., 1990) and may be involved in nucleolar localization of Tat (Hauber et al., 1987; Pearson et al., 1990; Ruben et al., 1989). Tat belongs to a family of RNA binding proteins that contain an arginine-rich motif for binding to specific RNA targets (Lazinski et al., 1989). Proteolytically cleaved carboxy-terminal fragments of Tat (Tfr) spanning the basic domain bind TAR RNA and a shortened form of TAR (ΔTAR, Figure 2) with subnanomolar affinity (Weeks et al., 1990).

Although the precise mechanism by which Tat stimulates gene expression remains unsolved, Tat effects occur primarily at the transcriptional level in mammalian cells (Rice & Matthews, 1988). Tat effects at the level of translation cannot be ruled out, however, since these predominate in *Xenopus* oocytes (Braddock et al., 1989, 1991). Tat requires specific regions of the promoter, including the NF-κB and Sp1 DNA sequences, for efficient transactivation (Berkhout et al., 1990; Kamine et al., 1991; Southgate & Green, 1991). Tat functions in part to target and bind TAR RNA, through which it is brought into proximity with the transcription complex (Gait & Karn, 1993; Weeks & Crothers, 1993b). Indeed, Tat fusion proteins containing heterologous RNA or DNA binding domains, upon binding to their cognate targets, are capable of transactivation in vivo (Selby & Peterlin, 1990; Southgate et al., 1990).

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There is evidence that Tat affects both the initiation (Laspia et al., 1989; Peterlin et al., 1986) and elongation (Feinberg et al., 1991; Kao et al., 1987; Laspia et al., 1993) steps of transcription. An accumulation of short, incompletely elongated mRNAs in Tat-defective viruses are efficiently elongated upon the addition of exogenous Tat (Kao et al., 1987). Tat also stimulates the transcriptional read-through of distal terminator sequences in vitro (Graeble et al., 1993). The emerging consensus is that Tat functions mainly as a promoter-specific elongation factor that modifies the transcription complex upon binding to TAR RNA. In addition, Tat may function as a processivity factor that enhances the ability of RNA polymerase to elongate transcripts over longer distances (Kato et al., 1992).

Much is known about the TAR RNA sequence and structural requirements for specific interaction with Tat. Modification interference experiments mapped the TAR RNA contact site to the trinucleotide bulge region and adjacent base pairs (Weeks et al., 1990). For sequence-specific interaction, the two base pairs above the bulge (G26−C39 and A27−U38) as well as a bulge of at least two nucleotides with a uridine residue at the base of the bulge are required (Churcher et al., 1993; Weeks & Crothers, 1991). Several phosphate groups adjacent to the bulge, including P21 (between G21 and A22), P22, and P40, are essential for RNA recognition by Tat, as indicated by interference experiments (Calnan et al., 1991b; Churcher et al., 1993) and methylphosphonate substitutions (Pritchard et al., 1994).

The bulge structural element of TAR is critical for Tat recognition. Reaction of TAR with diethyl pyrocarbonate (DEPC) demonstrated that the trinucleotide bulge acts to make the normally deep, narrow major groove of canonical (A-form) double-helical RNA more accessible (Weeks & Crothers, 1991). Enhanced major groove accessibility of RNA at helix termini lends support to major groove recognition as a common theme in protein−RNA interactions (Weeks & Crothers, 1993a). The bulge in TAR causes a retardation in gel mobility due to local bending of the helix axis (Bhattacharyya et al., 1990). Furthermore, the TAR sequence context adjacent to the bulge (purines on one strand and pyrimidines on the other strand) results in a more pronounced kinking of the helix axis rather than a randomly chosen sequence (Riordan et al., 1992).

Recent studies by Frankel and co-workers delineate a specific role for arginine in the interaction between Tat and TAR (Frankel, 1992b). Short peptides from the basic region of Tat or homopolymers of arginine specifically bind to TAR (Calnan et al., 1991a). Activities of Tat basic region mutants demonstrate that a basic region composed entirely of arginine or a single central arginine is sufficient to reproduce wild-type transactivation levels (Calnan et al., 1991b). In contrast, peptides with nine lysines bind TAR poorly (Calnan et al., 1991b), and mutated Tat with a basic region composed entirely of lysine has very low transactivation activity (Calnan et al., 1991b). Experiments using an L-arginine affinity column show that L-arginine can compete with a Tat-derived peptide for TAR binding, whereas L-lysine and certain analogs of arginine cannot (Tao & Frankel, 1992). Circular dichroism studies indicate that binding of arginine or guanidine induces the same conformational change in TAR as single arginine containing peptides (Tan & Frankel, 1992). These changes in CD spectra were not seen with L-lysine or TAR mutants that abolished specific binding (Tan & Frankel, 1992). NMR studies of a TAR−argininamide complex confirm that TAR has a specific arginine binding site and suggest a model for the Tat−TAR interaction (Puglisi et al., 1992). Upon arginine binding, TAR undergoes a conformational change wherein the uridine at the base of the bulge (U23) forms a triple with the essential A27−U38 base pair above the bulge (Puglisi et al., 1992). Base triple formation was verified through a series of triple mutants, including one containing an isomorphous base triple ($C23^{+}$· G27−C38) (Puglisi et al., 1993).

The sequence requirements within the Tat basic region that are critical for specific binding to TAR RNA are less clearly defined. In vivo studies indicate that at least five basic residues are required for full transactivation (Delling et al., 1991; Subramanian, et al., 1991; Tao & Frankel, 1993). Besides the arginine at position 52, which mediates specific complex formation with TAR, there is certainly some degree of sequence flexibility (arginine vs lysine vs another amino acid) at other positions in the basic region (Tao & Frankel, 1993). In fact, the basic region of Rev can functionally substitute for the basic region of Tat (Subramanian et al., 1990). In addition to an overall charge of the basic region, the exact charge distribution is also important. Both transactivation activities and specific RNA binding affinities of mutant Tat proteins reveal differential effects of removing a positive charge, depending upon its sequence context within the basic region (Tao & Frankel, 1993). The lysine residue at position 50 may provide an important RNA contact since a serine to lysine change at the analogous position in SIV Tat results in a 3−4-fold increase in its activity on the HIV promoter to levels comparable to that of HIV-1 Tat (Tao & Frankel, 1993).

We have studied the thermodynamics and kinetics of complex formation between TAR RNA and a set of 24 residue Tat-derived peptides containing one or two arginines as a means to screen these peptides as possible structural models for the wild type interaction. Although none of the Tat-derived peptides will be useful as models of wild-type, we show that at least three arginines within the basic region of Tat are necessary to reproduce the dissociation kinetic behavior of the wild-type complex. Through CD studies, we demonstrate that the CD signal changes upon complex formation are primarily due to changes in TAR RNA conformation.

MATERIALS AND METHODS

Oligoribonucleotides and Peptides. DNA templates for in vitro transcription were synthesized on an Applied Biosystems oligonucleotide synthesizer and purified by denaturing gel electrophoresis. Oligoribonucleotides were prepared from in vitro transcription reactions using T7 RNA polymerase (Milligan et al., 1987) and purified on 15−20% denaturing gels. Peptides were chemically synthesized at the Biotechnology Resource Laboratory of the W. M. Keck Foundation at Yale University Medical School on an Applied Biosystems Model 430A peptide synthesizer using standard procedures. The peptides were purified by reverse phase HPLC and characterized by mass spectrometry. Peptide concentration was determined through amino acid analysis, and stock solutions were stored at −20 °C.

Apparent Dissociation Constants. Peptides were titrated against 5′ [γ-^{32}P]ΔTAR RNA in 10 μL binding reactions

[10 mM Tris (pH 7.5), 70 mM NaCl, 0.2 mM EDTA, 5% glycerol, and 0.01% Nonidet P-40 (Shell Chemicals)] at room temperature. Reactions were loaded onto running native gels [10% (w/v) acrylamide and 75:1 acrylamide/bis(acrylamide) in 45 mM Tris−borate and 1 mM EDTA at 15 °C] and electrophoresed for 1 h at 25 V/cm. Native gels for the dissociation kinetics, association kinetics, and competition assays were run under the same conditions. Apparent dissociation constants (K_d) were estimated from

$$\Theta = \frac{[\text{peptide}]}{[\text{peptide}] + K_d} \quad (1)$$

where Θ is the percentage of bound radiolabeled probe.

Relative peptide binding affinities were also estimated through competition experiments with a longer carboxy-terminal Tat peptide, Tfr38 (Weeks et al., 1990). Radiolabeled ΔTAR was incubated with Tfr38 for 20 min. Reaction aliquots (10 μL) were added to increasing amounts of Tfr24-derived peptides in a 5 μL volume. After an incubation of 1 h, reactions were loaded onto a running native gel. The partitioning of ΔTAR between Tfr38- and Tfr24-derived peptides was quantitated with a betascope utilizing the mobility difference between the two complexes. The data were fit to the competition equations detailed in the following.

Dissociation Kinetics. Peptide−ΔTAR complexes were prepared under standard binding conditions. Peptide concentrations were sufficient for the complexation of 40−70% of radiolabeled ΔTAR. At time zero, unlabeled ΔTAR was added to a final concentration of 20−80 nM and the reaction was mixed with gentle pipetting. Aliquots were withdrawn at appropriate times and loaded onto a running native gel. Dried gels were analyzed using a betascope blot analyzer (Betagen Co., Waltham, MA). Dissociation rates were obtained from a best fit to

$$\frac{[\text{complex}]}{[\text{complex}]_{t=0}} = Ae^{-k_1 t} + Be^{-k_2 t} \quad (2)$$

Some of the peptide−ΔTAR dissociation curves fit a single-exponential function, as well as the sum of exponential functions in eq 2. Consequently, experimental half-lives are reported to enable comparison among all of the peptide−ΔTAR complexes.

Association Kinetics. Radiolabeled ΔTAR was added to peptide in 10 μL reactions at time zero. At the appropriate times, reaction aliquots were added to an excess of unlabeled competitor ΔTAR RNA in a 5 μL volume. These quenched reactions were loaded onto a running native gel after incubation for 2−3 min. Therefore, the quenching assay can only measure the association of complexes that have a half-life for dissociation that is long compared to the incubation time. The association data were fit to the following expression:

$$\frac{[\text{complex}]_t}{([\text{RNA}]_t + [\text{complex}]_t)} = 1 - e^{-k_a' t} \quad (3)$$

where k_a' is the pseudo-first-order rate constant. Since association is shown to be a bimolecular process, the second-order-rate constant, k_a, is obtained by dividing k_a' by the peptide concentration.

Competition Assay. Peptides were titrated against 0.5 nM ΔTAR spiked with 5′ [γ -^{32}P]ΔTAR RNA to establish a concentration at which 40−70% of the labeled RNA was bound when subjected to native gel electrophoresis. Competition experiments were performed in 15 μL volumes and included increasing concentrations of unlabeled competitor RNA. Peptide was added to reactions containing competitor RNA and radiolabeled ΔTAR, and the solutions were incubated at room temperature for 75 min before being loaded onto a running native gel. Bands corresponding to bound and free radiolabeled probe were quantitated using a betascope blot analyzer. The data were fit to the Linn and Riggs equation (Linn & Riggs, 1972), which describes the competitive binding of two ligands to a peptide. The fractional saturation of radiolabeled probe, Θ, is

$$\Theta = \frac{P_t(1 - \Theta)}{K_T[(1 + C_t)K_C] + T_t(1 - \Theta)} \quad (4)$$

where P_t, T_t, and C_t, are the concentrations of peptide, radiolabeled probe, and competitor RNA, respectively. The dissociation constants for ΔTAR−peptide and competitor RNA−peptide complexes are denoted K_T and K_C, respectively. Solution of eq 4 for Θ gives the physically relevant root,

$$\Theta = \frac{1}{2T_t}\{K_T + (K_T/K_C)C_t + P_t + T_t - \sqrt{[K_T + (K_T/K_C)C_t + P_t + T_t]^2 - 4T_tP_t}\} \quad (5)$$

A competition curve, $\Theta = f(C_t)$, may be fit for the best value of K_C using a nonlinear least-squares method. The apparent relative dissociation constant, K_{rel}, is the ratio of competitor to wild-type dissociation constants, $K_{rel} = K_C/K_T$, where a larger K_{rel} value implies weaker mutant binding.

Circular Dichroism Spectroscopy. Circular dichroism spectra were measured using an Aviv Model 62DS spectrometer. Spectra were recorded from 320 to 200 nm and averaged over five scans, with a 1 s averaging time at each wavelength. Samples were prepared in 10 mM potassium phosphate buffer (pH 7.5) and 10 mM NaCl. Spectra were recorded using a square quartz cuvette with a 2 mm path length at 25 °C. To ensure that the concentration of RNA was the same between samples of ΔTAR RNA and ΔTAR RNA−peptide complex, a solution of RNA was made and split into portions. An equal volume of either buffer or peptide was then added, allowing for direct subtraction of the spectra. The peptide and ΔTAR RNA concentrations used for all CD spectroscopy experiments are as follows: [peptide] = 4.8 μM, [ΔTAR RNA] = 5.3 μM, and [argininamide] = 3 mM.

RESULTS

Design of Tfr24-Derived Peptide Sequences. Peptides were synthesized from the context of a wild-type Tfr24 sequence, with mutations localized in the basic region. Tfr24-derived peptide sequences contain one or two arginines, with arginine to lysine changes at wild-type arginine positions to preserve overall charge. Peptides are named

Tfr24 (wildtype) RKKRRQRRRPPQGSQTHQVSLSKQ

position	49	50	51	52	53	54	55	56	57
Tfr24	Arg	Lys	Lys	Arg	Arg	Gln	Arg	Arg	Arg
R52	Lys	Lys	Lys	Arg	Lys	Gln	Lys	Lys	Lys
R52K54	Lys	Lys	Lys	Arg	Lys	Lys	Lys	Lys	Lys
R53	Lys	Lys	Lys	Lys	Arg	Gln	Lys	Lys	Lys
R52R53	Lys	Lys	Lys	Arg	Arg	Gln	Lys	Lys	Lys
R52R53K54	Lys	Lys	Lys	Arg	Arg	Lys	Lys	Lys	Lys
R55	Lys	Lys	Lys	Lys	Lys	Gln	Arg	Lys	Lys
R56	Lys	Lys	Lys	Lys	Lys	Gln	Lys	Arg	Lys

FIGURE 1: Tfr24-derived peptide sequences containing 24 amino acids with one or two arginines. Mutations are restricted to the N-terminal basic region. Peptide sequences are named according to the position(s) of arginine and other sequence changes from the wild-type sequence. In general, arginines in the wild-type sequence have been replaced by lysines in order to preserve overall charge.

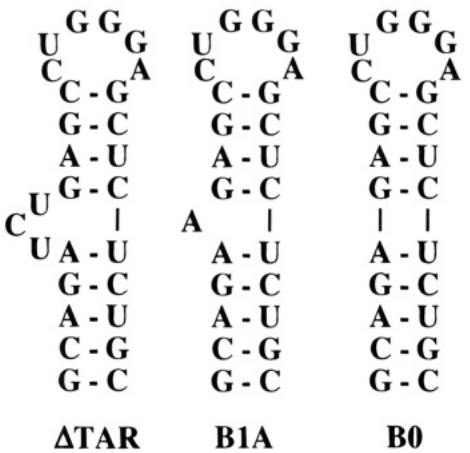

FIGURE 2: Sequence and secondary structures of ΔTAR RNA and ΔTAR bulge mutants used in competition experiments. Positions of ΔTAR are numbered (5′ to 3′) from G18 to C44, with position +1 denoting the transcription start site. The ΔTAR bulge mutants include B1A and B0, which contain a single A bulge and no bulged nucleotide, respectively. The bulge mutants, B1A and B0, bind Tfr38 with relative binding constants 22- and >40-fold greater than that of wild type, respectively (Weeks & Crothers, 1991).

according to the position(s) of arginine within the sequence in addition to changes from the wild-type sequence (Figure 1). Several studies suggest that arginines in the center of the basic region of Tat (residues 49−57) are more important for TAR RNA binding and transactivation than those at the termini (Calnan et al., 1991a,b) . Peptides with one arginine are R52, R52K54, R53, R55, and R56. Peptides with two arginines are R52R53 and R52R53K54. The sequences R52K54 and R52R53K54 were made to evaluate the role of glutamine and the effect of an additional positive charge at position 54.

Tfr24-Derived Peptides Specifically Bind to ΔTAR RNA. Titration of Tfr24-derived peptides to ΔTAR RNA (Figure 2) in a mobility shift assay results in a single band corresponding to a (1:1) peptide−ΔTAR complex. The various peptide−ΔTAR complexes exhibit subtle differences in gel mobility (Figure 3). The decreased gel mobility of R52K54 and R52R53K54 complexes may be explained on the basis that these peptides contain an additional positively

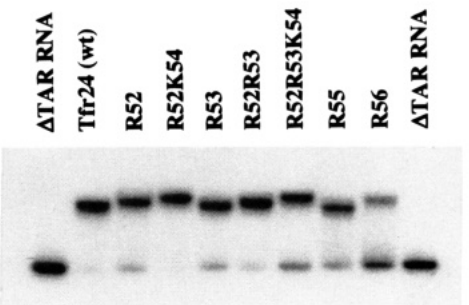

FIGURE 3: Tfr24-derived peptide−ΔTAR RNA complexes. The autoradiogram shows free and bound ΔTAR RNA resolved by native gel electrophoresis. The outside lanes correspond to ΔTAR RNA in the absence of peptide. All peptides are at a concentration of 70 nM.

Table 1: Apparent Dissociation Constants of Tfr24-Derived Peptide−ΔTAR RNA Complexes[a]

peptide	apparent K_d (nM)
Tfr24	0.4
R52	0.9
R52K54	0.7
R53	3.5
R52R53	0.5
R52R53K54	1.8
R55	>50
R56	>100

[a] These values are reported with a cautionary note. We show that the gel band shift titration methods (see Materials and Methods) used to obtain these data cannot accurately determine subnanomolar binding affinities.

charged lysine residue. Other subtle variations in complex mobility that cannot be explained by molecular weight (Tfr24 vs R52, for example) are presumably due to conformational differences.

Apparent dissociation constants for the peptide-ΔTAR complexes estimated from band shift titrations are summarized in Table 1. However, we demonstrate in the following that these methods are not capable of accurately determining subnanomolar peptide−ΔTAR binding affinities. We find that the direct titration method seriously underes-

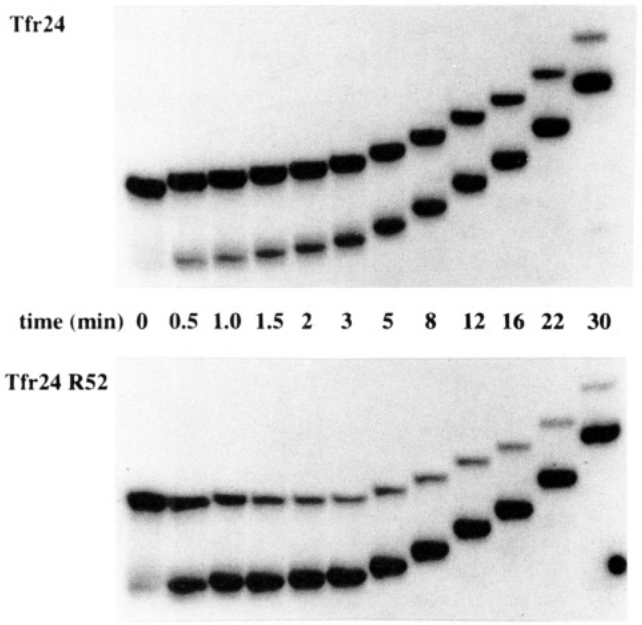

FIGURE 4: Autoradiogram of Tfr24 and Tfr24R52 peptide−ΔTAR RNA complexes. The first lanes are the peptide−ΔTAR RNA complexes in the absence of cold ΔTAR RNA competitor. Subsequent lanes are reaction aliquots loaded onto a native gel after competitor addition (20 nM) at the times shown.

timates both binding affinity and variations in affinity among members of the set. On a qualitative basis, we nevertheless can conclude that peptides that contain an arginine at position 52 have ΔTAR RNA binding affinity that is most similar to the wild-type peptide, Tfr24. On the basis of band shift titrations, if a single arginine is placed at positions 53, 55, and 56, the binding affinity decreases by 9-, >125-, and >250-fold, respectively. This trend in binding affinity is paralleled by transactivation levels of basic region mutants of Tat protein in HeLa cells (Calnan et al., 1991b). Clearly, the arginine at position 52 plays a more critical role in peptide binding affinity than those at positions 53, 55, and 56.

Kinetic Stability of Tfr24-Derived Peptide−ΔTAR RNA Complexes. In order for a peptide with sequence modifications to serve as an acceptable model of the wild type in its interaction with ΔTAR RNA, the resulting peptide−ΔTAR complex must have thermodynamic and kinetic stabilities that are comparable to those of the wild type. Kinetic stability of the complexes has been assessed through examination of dissociation and association kinetics. Native gel electrophoresis was used to follow the dissociation of preincubated peptide−ΔTAR complexes after the addition of unlabeled ΔTAR RNA competitor. Dissociation of the complexes was measured over a range of ΔTAR RNA competitor concentrations.

Tfr24-derived peptide−ΔTAR complexes are much less stable to dissociation than the wild-type complex. Typical dissociation profiles of Tfr24 and R52 complexes with 20 nM unlabeled competitor (Figures 4 and 5) illustrate the striking differences in dissociation behavior of peptide−ΔTAR complexes between peptides with one or two arginines and the wild type. The wild-type complex exhibits monophasic dissociation behavior with a half-life of 9.2 min. In contrast, the R52−ΔTAR complex has a half-life of 0.6 min and a biphasic dissociation profile including fast and slow components. With the exception of the R52R53K54 complex, which has an intermediate stability, the other Tfr24-derived peptide−ΔTAR complexes have half-lives of ≤2 min (at [competitor] = 20 nM).

The experimental lifetimes of the peptide−ΔTAR complexes are summarized in Table 2. In terms of their half-

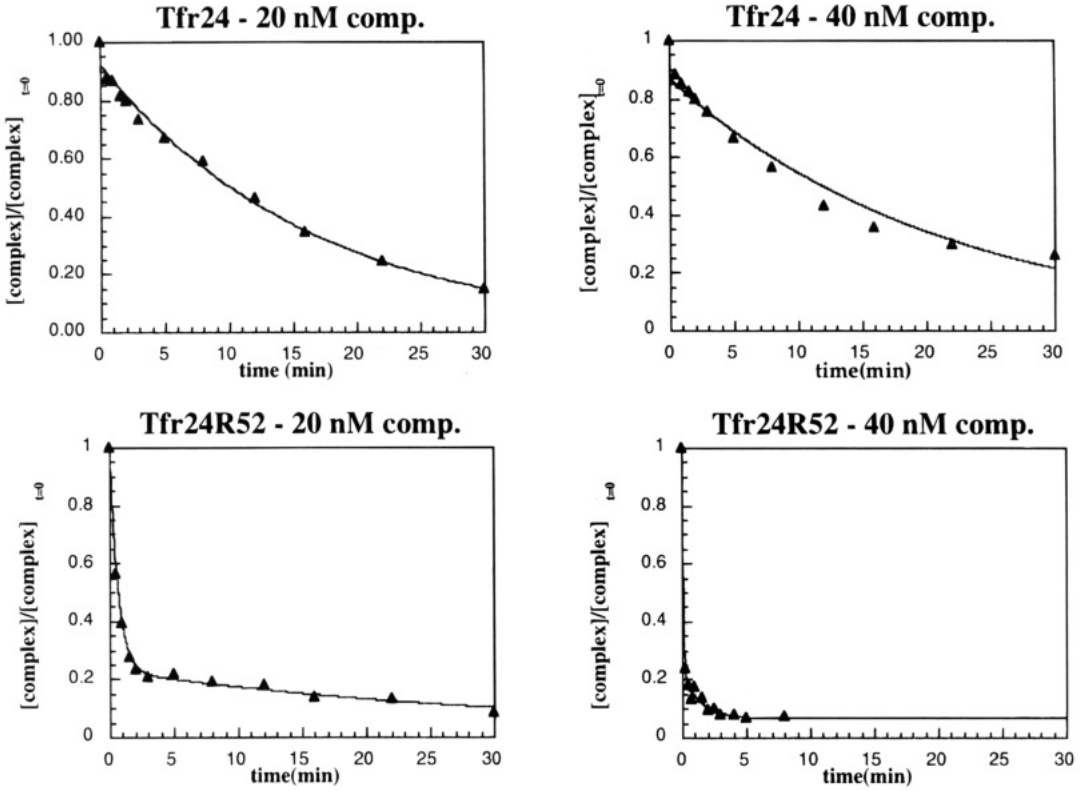

FIGURE 5: Plots of dissociation kinetics. The data in Figure 4 are plotted as the fraction of initial complex before competitor addition vs time after competitor addition. Dissociation curves for Tfr24 and R52 peptide−ΔTAR complexes are shown at competitor concentrations of 20 and 40 nM.

Table 2: Summary of Experimental Peptide−ΔTAR Complex Lifetimes

peptide	competitor concn (nM)	$t_{1/2}$ (min)
Tfr24	20	9.2
	40	9.9
	60	11.4
	80	2.8
R52	20	0.6
	30	<0.13
	40	<0.083
	60	<0.083
R52K54	20	2.0
	30	3.0
	40	1.6
	60	1.3
R53	25	0.18
	30	0.15
	40	0.12
	60	0.083
R52R53	20	2.0
	30	1.2
	40	0.8
	60	0.6
R52R53K54	25	7.0
	30	4.9
	40	2.3
	60	2.2

lives, the complexes fall in the order Tfr24 > R52R53K54 > R52K54 > R52R53 > R52 > R53. R55 and R56 do not form complexes that are stable to gel electrophoresis. For the R52, R53, R52R53, and R52R53K54 complexes, lifetimes decrease with increasing competitor concentration. The dissociation of these complexes can be explained by a facilitated transfer mechanism, where dissociation is first-order in competing RNA (Fried & Crothers, 1984). Tfr24 and R52K54 complexes show a higher order dependence on competitor concentration. This observation contrasts with a previously reported result with a 38 amino acid wild-type carboxy-terminal peptide, Tfr38, where the dissociation rate increases linearly with increasing competitor concentration (Weeks & Crothers, 1991).

Kinetic stabilities of peptide-ΔTAR complexes cannot be predicted from the apparent dissociation constants estimated from band shift titrations in Table 1. While the R52 complex has binding affinity comparable to that of wild type, its dissociation is much more rapid. In another case, R52R53 has a higher ΔTAR RNA binding affinity than R52R53K54, although the half-life of the R52R53K54 complex is 2.5-fold longer than that of R52R53. In comparing the following pairs of complexes, R52/R52K54 and R52R53/R52R53K54, the effect of an additional positive charge is to increase the lifetime of the complex. Since peptides with net charge identical to that of wild type have much shorter half-lives, additional arginines within the basic region are needed to reproduce wild-type kinetic stability.

The distinct dissociation behaviors of the various peptide−ΔTAR complexes imply that these complexes adopt conformations or interactions different from wild type. Although none of the peptides will serve as useful structural models of the wild-type complex, their behavior indicates that at least three of the six arginines in the basic region of Tat play critical roles in the kinetic stability of the Tat−TAR complex.

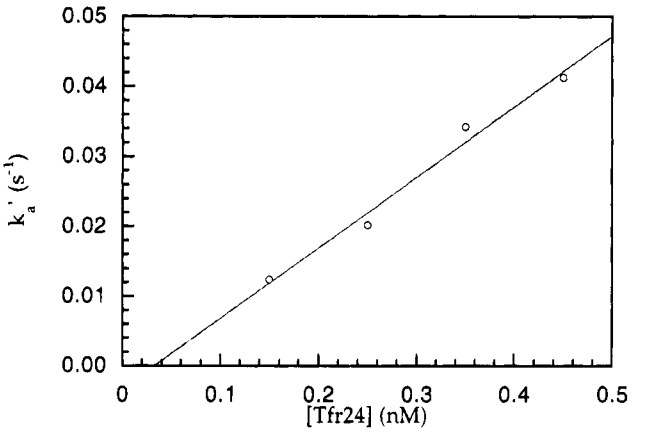

FIGURE 6: Linear dependence of the pseudo-first-order association rate on Tfr24 concentration. The association rate is given by the slope of the line, 1×10^8 M^{-1} s^{-1}.

Table 3: Experimental and Predicted Association Rate Constants for Peptide−ΔTAR Complexes

peptide	k_{on} (M^{-1} s^{-1}), predicted	k_{on} (M^{-1} s^{-1}), expml	K_d (pM), from measured k_{on} and k_{off} values
Tfr24	3.3×10^6	7.9×10^7	16
R52	2.1×10^7	*a*	
R52K54	8.3×10^6	*a*	
R53	1.8×10^7	*a*	
R52R53	1.2×10^7	*a*	
R52R53K54	9.4×10^5	9.1×10^7	18

[a] Dissociation of these complexes is too rapid to enable the measurement of association rate constants.

Association Kinetics. Association rates were determined using a quenching protocol (see Materials and Methods). This approach ensures that complexes detected by gel electrophoresis were formed prior to loading on the gel. Measured association rate constants for Tfr24− and R52R53K54−ΔTAR complexes are 7.9×10^7 and 9.1×10^7 M^{-1} s^{-1} at peptide concentrations of 0.25 and 2 nM, respectively. Association rate constants of the wild-type complex were measured over a range of Tfr24 concentrations. Since the pseudo-first-order rate constant, k_a', increases linearly with increasing peptide concentration (Figure 6), association is a second-order process. The association rate constant is given by the slope of the line, which is approximately 1×10^8 M^{-1} s^{-1}. Division of the dissociation rate constant measured in the limit of low competitor concentration by the second-order association rate constant yields an equilibrium dissociation constant, K_d, of about 16 pM.

Predicted association rate constants may be calculated from the dissociation rate divided by the apparent dissociation constant from the gel titration. Experimental and predicted association rate constants are summarized in Table 3. Predicted association rate constants are 1−2 orders of magnitude slower than experimentally determined values. This discrepancy implies that the band shift gel titration method to estimate apparent dissociation constants is not able to determine the relative order of binding affinities or absolute values of K_d in the subnanomolar range. Apparent dissociation constants calculated from measured association and dissociation rate constants are on the order of 10^{-11} M. Therefore, binding affinities determined from gel titration methods are underestimated 25−100-fold. We suspect that this is due to the requirement for a threshold concentration of peptide and RNA before complexes can be observed.

Table 4: Relative ΔTAR RNA Binding Affinities of Tfr24-Derived Peptides Determined through Competition with Tfr38

peptide	$D_{1/2}$ (nM)	$D_{1/2}t_{1/2}$ (nM· min)
Tfr24	2.3	12
R52	16	8.4
R52K54	8.0	16
R53	40	7.2
R52R53	15	30
R52R53K54	4.0	28

Peptide Competition. Competition experiments with Tfr38 were used to measure relative binding affinities between the peptide−ΔTAR complexes. Table 4 summarizes $D_{1/2}$ values, or the concentration of peptide required to compete away 50% of the original Tfr38−ΔTAR complex. The R55 and R56 peptides were not able to compete for the Tfr38−ΔTAR complex, even at concentrations greater than 1 μM. For the other peptides, the trend in binding affinities parallels that of kinetic stabilities. The relative binding affinities determined by competition (Table 4) emphasize the inability of the gel titration method to distinguish between 7-fold differences in binding affinity below the nanomolar range (Tfr24 vs R52, Tables 1 and 4). The product of the binding affinity and the complex lifetime for each complex is a constant within experimental error (Table 4), implying that the association rate constant is essentially invariant at about 1×10^8 M^{-1} s^{-1}. Note that competition methods, which can be carried out at concentrations well above K_d, appear to be the most reliable mode of quantitation using gel electrophoretic analysis.

Relative Binding Affinities for ΔTAR Bulge Mutants. The bulge nucleotides of ΔTAR RNA are critical determinants of specific binding to wild-type Tat peptides. At least two bulge nucleotides are required for sequence specificity in binding, whereas one bulge nucleotide of any sequence is sufficient for a nonspecific interaction (Weeks & Crothers, 1991). In the case of the Tfr38−ΔTAR complex, the relative binding constants for a single A bulge mutant (B1A) and a bulgeless mutant (B0) of ΔTAR RNA (Figure 2) are 22- and > 40-fold lower than that of wild type, respectively (Weeks & Crothers, 1991). Competition experiments have been used to address whether the Tfr24-derived peptide sequences discriminate between these ΔTAR RNA bulge mutants, as well as the wild-type peptide, Tfr24.

Among all of the peptide−ΔTAR complexes, the Tfr24R52R53K54 complex has the longest lifetime aside from the wild type. Competition experiments (as described in Materials and Methods) reveal that the Tfr24R52R53K54 complex has a comparable but measurably lower ability to distinguish between ΔTAR RNA bulge mutants (Figure 7). The relative binding affinities of all peptide−ΔTAR complexes for ΔTAR RNA bulge mutants are summarized in Table 5.

Peptides with one arginine (R52, R52K54, and R53) do not interact specifically with the trinucleotide bulge in ΔTAR. Relative binding affinities to B1A RNA are 2.5-fold lower in the case of R52 and R52K54 complexes. The R53 peptide, having the poorest discrimination ability, binds equally well to B1A and ΔTAR RNAs. The addition of a positive charge (R52K54, R52R53K54) clearly increases discrimination over the bulgeless mutant, B0.

There is good correlation between the binding affinity (determined by competition) of a particular peptide−ΔTAR

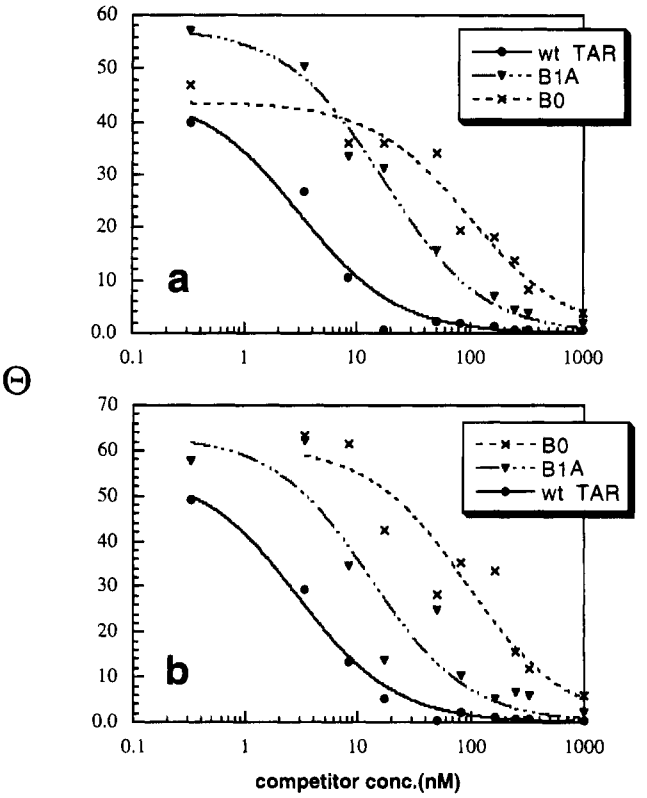

FIGURE 7: Competition titrations for (a) Tfr24 and (b) Tfr24R52R53K54. The data are plotted as the fraction of radiolabeled probe bound (Θ) vs the concentration of unlabeled competitor.

Table 5: Relative Binding Affinities for ΔTAR RNA Bulge Mutants Determined by Competition

peptide	$D_{1/2}^{wt}$ (nM)	$D_{1/2}^{B0}$ (nM)	$D_{1/2}^{B1A}$ (nM)	K_{rel}^{B0}	K_{rel}^{B1A}
Tfr24	4	135	42.5	34	11
R52	8	34	20	4.3	2.5
R52K54	3.7	100	9	27	2.4
R53	300	1200	300	4	1
R52R53	4	40	17.5	10	4.4
R52R53K54	2.5	65	20	26	8

complex and that peptide's ability to distinguish between bulge mutant and wild-type RNAs. Furthermore, there is good correspondence between bulge discrimination ability and peptide−ΔTAR dissociation rate. The results presented here are consistent with previous kinetic data collected in the Tat peptide system (Weeks & Crothers, 1992). These studies demonstrate that a shorter peptide, Tfr14, does not discriminate as well between specific and nonspecific sites as a longer peptide, Tfr38 (Weeks & Crothers, 1992). In addition, Tfr14 dissociates from ΔTAR RNA approximately 5 times faster than Tfr38 (Weeks & Crothers, 1992). Taken together, these findings clearly indicate that kinetic stability is an important determinant of binding specificity in basic peptide−RNA interactions.

Circular Dichroism Spectroscopy. Carboxy-terminal fragments of Tat adopt a random coil conformation in solution (Figure 8a). Upon binding to ΔTAR RNA, they fold into a conformation that remains unknown. In comparing the ΔTAR RNA and the Tfr24−ΔTAR complex CD spectra, there are changes below 240 nm and centered around 260 nm (Figure 8b). These data are consistent with previous CD studies of TAR RNA and Tat peptides (Calnan et al., 1991a; Loret et al., 1992). The ellipticity decrease at 260 nm has been assigned to a change in ΔTAR RNA upon complex

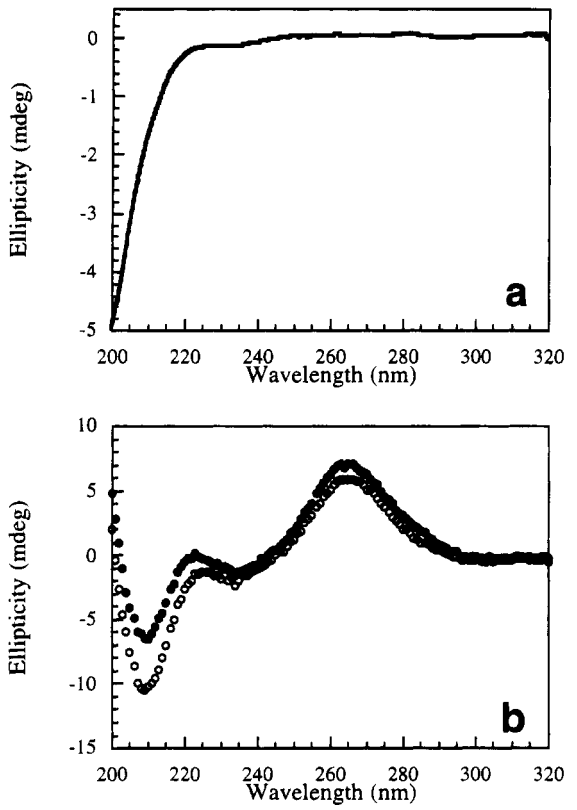

FIGURE 8: CD spectra of (a)Tfr24 (b) ΔTAR RNA (●) and Tfr24−ΔTAR complex (○). The concentrations of Tfr24 and ΔTAR RNA are 4.8 and 5.3 μM, respectively.

formation (Tan & Frankel, 1992). Changes in the CD spectra of nucleic acids are thought to be due to perturbations in base stacking (Riazance et al., 1985). A decrease in base stacking is consistent with recent NMR studies of TAR RNA and the TAR−argininamide complex (Puglisi et al., 1992).

Solutions of ΔTAR and peptide−ΔTAR complex for CD spectroscopy were prepared (as described in Materials and Methods) so as to allow for direct subtraction of CD spectra. Subtraction of ΔTAR RNA spectra from peptide−ΔTAR complex CD spectra provide difference spectra that represent the summation of changes in peptide and ΔTAR RNA structure that occur upon complex formation. Difference CD spectra are a means to compare these changes for all peptide−ΔTAR complexes (Figure 9).

The CD difference spectrum for Tfr24 has distinct bands centered at 215, 260, and 280 nm. This difference spectrum is similar to that of a shorter wild-type peptide, Tat 47−58 (Calnan et al., 1991a). The difference spectra for all of the other peptide-ΔTAR complexes look quite distinct from that of wild type. The R52 difference spectrum is the most similar to that of wild type; however, there are clear distinctions in the band intensity at 215 nm and in the ratio of intensities at 260 and 280 nm. Aside from R52, there is no appreciable band at 260 nm in the difference spectra for the complexes, indicating that the other peptides do not induce the same conformational change in ΔTAR RNA as wild type. These results confirm that the Tfr24-derived peptides with one and two arginine residues of this study will not serve as good structural models of the wild-type peptide in binding to ΔTAR RNA.

Upon arginine binding, ΔTAR RNA undergoes a conformational change involving the formation of a base triple to form a specific binding cleft for arginine (Puglisi et al., 1992). In order to compare the changes in CD spectra upon complex formation with the Tfr24-derived peptides of this study, we have studied the argininamide−ΔTAR RNA complex using CD spectroscopy. Unlike the CD spectra of L-lysine and L-arginine (Tan & Frankel, 1992), the CD spectrum of a 3 mM solution of L-argininamide has a shallow minimum at 225 nm (Figure 10a). There are several changes in the CD spectra of ΔTAR RNA and the argininamide−ΔTAR complex (Figure 10b). Like the CD spectra of the peptide−ΔTAR complexes, there is a decrease in the amplitude of the maximum at 260 nm.

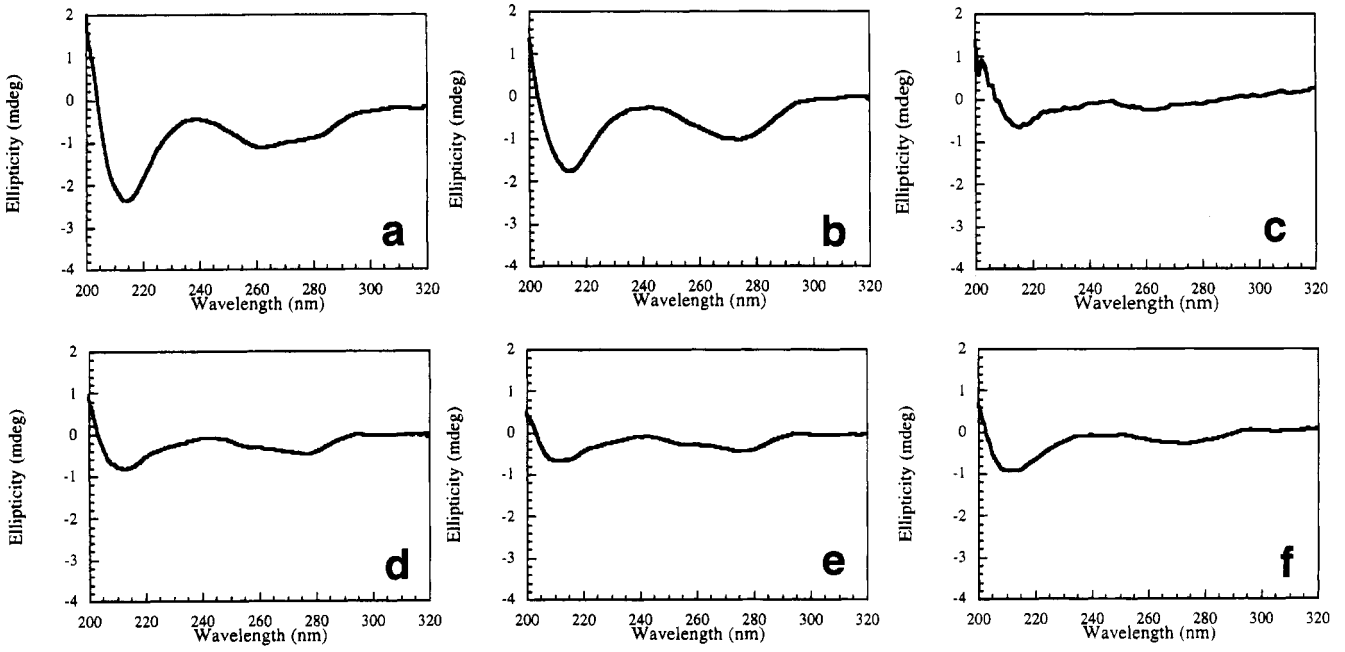

FIGURE 9: Difference CD spectra for peptide−ΔTAR RNA complexes: (a) Tfr24, (b) R52, (c) R52K54, (d) R52R53, (e) R52R53K54, and (f) R53. The difference spectra were obtained through subtraction of the CD spectra of ΔTAR RNA and of peptide alone from the peptide−ΔTAR complex spectrum. For all of the CD spectra of peptide-ΔTAR RNA complexes, the concentrations of peptide and ΔTAR RNA are 4.8 and 5.3 μM, respectively.

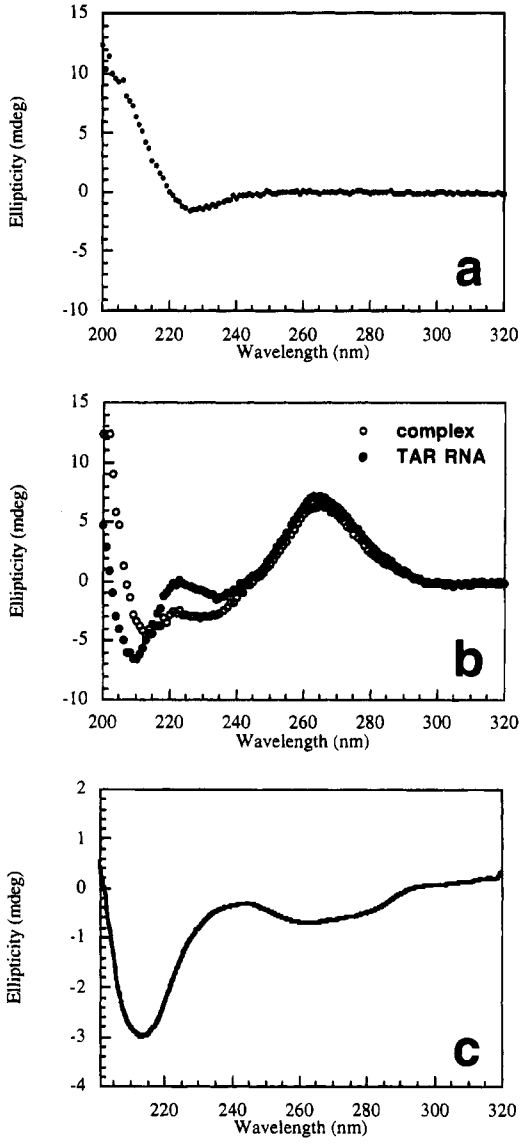

FIGURE 10: CD spectra of (a) argininamide, (b) argininamide−ΔTAR complex and (c) the difference CD spectrum for argininamide. The concentrations of argininamide and ΔTAR RNA are 3 mM and 5.3 μM, respectively.

Interestingly, the argininamide difference spectrum (Figure 10c) is most similar to the wild-type difference spectrum. The two CD difference spectra have similar amplitude ratios of bands centered at 215, 260, and 280 nm. These data indicate that the complex formed between a 3 mM solution of argininamide and ΔTAR RNA provides a more accurate structural model of the wild-type complex than any of the other peptide−ΔTAR complexes. These studies confirm that most of the CD changes that occur upon peptide−ΔTAR complex formation are due to alterations in ΔTAR RNA conformation. Multiple guanidinium groups in a bulk solution of argininamide are available for specific interactions with each molecule of TAR RNA. The similarity of the two difference spectra suggests that there may be several specific interactions between arginine side chains and ΔTAR RNA.

DISCUSSION

Tfr24-derived peptide−ΔTAR complexes are much less stable to dissociation than the wild-type complex. The kinetic stability of these complexes is in excellent correlation with both binding affinity and bulge discrimination ability. What then are the determinants of wild-type kinetic stability? In addition to a central arginine at position 52 and a certain overall positive charge within the basic region, other arginines in flanking positions provide specific contacts with TAR through either electrostatic interactions or hydrogen bonds. Transactivation levels for basic region Tat mutants do not translate into thermodynamic and kinetic stabilities for Tfr24-derived peptide−ΔTAR complexes. Apparently, transactivation levels can be affected similarly through the formation of a more transient complex than the wild type. Another possibility is that there are large differences between in vitro and in vivo Tat−TAR binding. Regions of Tat flanking the basic domain may be required for TAR RNA binding in vivo (Luo et al., 1993; Luo & Peterlin, 1993), although there is not yet sufficient structural evidence to support this idea. NMR studies of EIAV (equine infectious anemia virus) Tat suggest that N- and C-terminal amino acids come together with the basic region to form a positively charged cleft consisting of five arginines and four lysines (Sticht et al., 1993; Willbold et al., 1993, 1994).

The individual dissociation kinetics of the Tfr24-derived peptide−ΔTAR and wild-type complexes suggest a model for distinction between these systems. The wild-type complex may be more stable to dissociation than the other complexes, because the wild-type peptide is capable of stabilizing the complex by providing additional arginines to multiple specific binding sites. The other Tfr24-derived peptides do not contain enough arginines to saturate binding sites through a "chelate effect". The biphasic dissociation kinetics of the Tfr24-derived peptide−ΔTAR complexes further suggest that these peptides are binding to ΔTAR RNA in multiple conformations, possibly associated with occupancy of the different arginine binding sites by the one or two available arginines.

There is significant variability in absolute binding affinities for Tat peptide−TAR complexes derived from direct titration measurements, ranging from 60 pM to 300 nM in the literature (Churcher et al., 1993; Weeks & Crothers, 1991, 1992). Using association and dissociation rate constants, we estimate the absolute binding affinity of the Tfr24−ΔTAR complex to be appoximately 16 pM. Our results demonstrate that direct titration methodology cannot determine dissociation constants in the subnanomolar range. A threshold concentration apparently exists, below which complexes are not stable to gel electrophoresis. In such cases, the most effective way to estimate absolute binding affinity is through kinetic association and dissociation rates. However, relative binding affinities may be obtained from competition experiments using gel electrophoresis because concentrations can be kept above the threshold value.

The importance of RNA structure is established as a pervasive theme in the study of RNA−protein interactions. The formation of non-Watson−Crick base pairs in the Rev-RRE (rev response element) complex (Bartel et al., 1991; Battiste et al., 1994; Peterson et al., 1994) and the importance of hairpin loop secondary structure to the interaction between bacteriophage R17 coat protein and its RNA target (Romaniuk et al., 1987) are representative examples. Differences in CD spectra upon peptide−ΔTAR complex formation are mostly due to changes in ΔTAR RNA conformation. These CD changes are consistent with alterations in TAR RNA structure that have been observed in the TAR−argininamide

complex using NMR spectroscopy. The transition of Tfr24 and Tfr24-derived peptides from a random coil conformation to a bound form upon complexation with TAR RNA apparently does not produce a large perturbation in the CD signal.

In light of the similarities between the wild type and argininamide−ΔTAR difference spectra, it is likely that there are several arginine affinity sites on the surface of ΔTAR RNA with a specific preference for arginine over lysine. A relatively concentrated argininamide solution could provide enough unconstrained guanidinium groups to saturate these sites. In the Tfr24-derived peptides used in this study, guanidinium functionalities were not available in the numbers and positions necessary to induce the TAR RNA conformational changes observed with the wild-type peptide. Argininamide may be a useful model in terms of evaluating overall structural changes in TAR RNA upon complex formation, but it provides little insight into the bound conformation of Tat.

Arginine is ubiquitous at protein−RNA interfaces. This is substantiated in the crystal structures of the *Escherichia coli* glutaminyl-tRNA synthetase−$tRNA^{Gln}$ (Rould et al., 1989, 1991) and small nuclear ribonucleoprotein U1A−U1 small nuclear RNA (Oubridge et al., 1994) complexes. Indeed, there is a specific RNA binding site for arginine in the *Tetrahymena* intron, where it inhibits the self-splicing reaction through occupation of the G site (Yarus, 1988, 1989). Our results indicate, however, that one arginine is necessary but not sufficient for thermodynamic and kinetic stability comparable to that of the wild-type Tat−TAR complex. A feature common to RNA binding domains containing many arginine residues may be their inherent flexibility (Frankel, 1992a). In this way, basic RNA binding regions may be analogous to the acidic DNA binding domains of transcriptional activators (Ptashne, 1988; Sigler, 1988). Flexibility enables these domains to mediate nucleic acid interactions through the presentation of a specific electrostatic recognition surface while minimizing the energetic costs incurred upon binding to cognate targets.

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